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14. ABSTRACT We have previously identified seven low molecular weight compounds that inhibit the interaction between the c-Myc oncoprotein and its obligate heterodimerization partner, Max. This occurs as a consequence of the compounds binding directly to c-Myc and blocking its association with Max. These compounds thus prevent the growth of c-Myc over-expressing cells and inhibit their growth as in vivo tumors. However, they are of relatively low potency and cannot be considered to be clinically useful at the current time. Over the past year, we have begun to introduce structural modifications into one of these compounds and have developed a series of rules to explain the consequent structure-activity relationships. In one case, these rules have been applied in the design of small libraries of compounds in order to identify structural derivatives of the index compound. In this way, several "2nd generation" compounds with approx. 4-fold enhanced potencies have been developed. Over the next year, we aim to continue to improve the potencies of these compounds. In addition, using in vitro mutagenesis plus techniques that allow us to follow binding of these molecules to c-Myc, we will identify the precise amino acid residues in c-Myc necessary for this binding. These studies will set the stage for us to be able to link together two molecules, which bind to distinct sites of c-Myc, thus allowing us to attain synergistic protein binding and improved potency.

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Introduction

The c-Myc oncoprotein is a general transcription factors, which has been implicated in many human cancers, including those of the colon, breast, and prostate (1). We have previously identified seven low molecular weight compounds that prevent and/or disrupt the association between the c-Myc and its obligate heterodimerization partner, Max (2). We have also demonstrated a high degree of specificity for these compounds, as evidenced by their inability to dissociate other heterodimeric transcription factors (2). In all seven cases, these compounds inhibited the *in vitro* growth of mammalian cells that express c-Myc, but not of c-Myc -/- cells. Finally short-term, *in vitro* treatment of c-Myc-transformed fibroblasts with several of these compounds did not affect their viability but did reduce by >90% their subsequent ability to produce tumors in nude mice without any further treatment. The significant anti-tumor effects of these agents suggest that they, or related compounds, may be effective clinical agents. However, their low potency makes it unlikely that they will be useable in their current forms.

Body

Members of the Myc oncoprotein family are basic-helix-loop-helix-leucine zipper (bHLH-ZIP) transcription factors that are over-expressed in many human cancers (1). Specifically, c-Myc de-regulation is quite frequent in prostate cancer, occurring in as many as 50% of tumors, particularly those of advanced stage (1, 3). Recent evidence indicates that even short-term inhibition of c-Myc is sufficient to induce tumor regression in experimental animal models (4).

From a library of ca. 10,000 low molecular weight compounds, we have identified 7 that can prevent the association between c-Myc and Max. These compounds also inhibited the *in vitro* growth of mammalian cells expressing either normal or elevated levels of c-Myc, but did not inhibit the growth of c-Myc -/- "knockout" cells (ref. 2). In our original application, we proposed to extend this work by proposing four specific tasks:

Statement of original tasks

Task 1: to demonstrate directly that each of the previously identified compounds either prevents or disrupts c-Myc-Max heterodimerization.

Task 2: to conduct a series of in vivo studies aimed at determining whether these compounds can be effectively employed to treat c-Myc over-expressing tumors.

Task 3: to determine whether any of the compounds can be utilized in combination as a means of reducing toxicity and enhancing the antineoplastic effect.

Task 4: to employ computerized "data mining" techniques to determine whether other, as yet untested, but structurally related compounds might be better suited as potential therapeutic agents.

Key Research Accomplishments (2004-2005)

Most progress this year has been made on Tasks 1 and 4 and is summarized below

Task 1: to demonstrate directly that each of the previously identified compounds either prevents or disrupts c-Myc-Max heterodimerization.

In collaboration with our colleague, Dr. Steven J. Metallo (Georgetown University), we have observed that some of our originally identified parental compounds (10058, 10074-G5 and several others) are fluorescent and can de-polarize UV light to a degree that is related to the rate at which they move or "tumble" in solution. Binding of these molecules to c-Myc should reduce this movement and thus the degree to which de-polarization occurs (Fig. 1A). As seen in Fig. 1B, this proved to be the case. Moreover, titration studies indicated that both compounds bound to recombinant (His6)-c-Myc bHLH-ZIP domain with 1:1 stoichiometries (not shown). Control experiments further indicated that fluorescence de-polarization did not occur when the compounds were added to recombinant (His6)-Max (not shown). Taken together, these studies indicate that a simple fluorescence depolarization assay can be used to observe and quantify the binding of certain compounds to the bHLH-ZIP domain of c-Myc. Furthermore, they are consistent with the idea that these compounds bind exclusively to c-Myc and that, in doing so, they inhibit its ability to dimerize with Max.

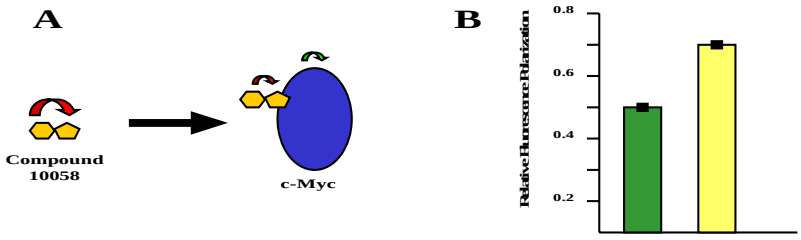

Fig. 1. (A). Compound 10058 depolarizes incident UV light partly as a function of its rate of movement (red arrow). Binding of the molecule to c-Myc, which has a much lower intrinsic rotational activity (green arrow) results in a reduction in 10058's fluorescence de-polarization potential. (B). Actual fluorescence profile of 10058 before and after the addition of purified recombinant (His6)-c-Myc bHLH-ZIP domain. The left hand dark green box indicates the relative fluorescence depolarization of 50 mM of compound 10058 in the absence of recombinant protein (avg. of 3 determinations +/- 1 S.E.). The right hand yellow box indicates the relative fluorescence of the same amount of 10058 in the presence of 50 μM of purified recombinant c-Myc protein. Note in the latter case that the loss of fluorescence de-polarization results in an increase in the amount of measured polarized light.

To independently assess the effects of c-Myc-Max compounds and to provide a means of examining the remaining non-fluorescent compounds, we conducted electrophoretic mobility shift assays (EMSAs) using purified, recombinant (His6)-tagged c-Myc and Max proteins (5). In the latter case, two isoforms of Max were used: Max (160), which homodimerizess and binds DNA, and Max (151), which homodimerizes but does not bind DNA (5). Both Max isoforms, however, do heterodimerize with c-Myc and bind DNA. c-Myc alone neither homodimerizes nor binds DNA alone. The target oligonucleotide consisted of a ^{32}P-end labeled 26 nt long palindromic oligonucleotide of sequence 5'-ggaagcagacCACGTGgtctgcttcc-3' (ref. 5), where the capitalized nucleotides indicate a consensus c-Myc binding site, or so-called "E-box". As seen in Fig. 2, (His6)-Max (160), showed strong binding. A mixture of c-Myc and Max (151) proteins revealed the presence of a new faster moving band indicating binding by c-Myc-Max heterodimers. Addition of the indicated Myc-Max compounds disrupted probe binding by the c-Myc-Max (151) heterodimer but not by Max (160) homodimers. These experiments are consistent with the idea that c-Myc-Max compounds interfere with Myc-Max heterodimerization but not with Max homodimerization. They are further consistent with those presented in Fig. 1 showing that some Myc-Max compounds bind directly to c-Myc monomers.

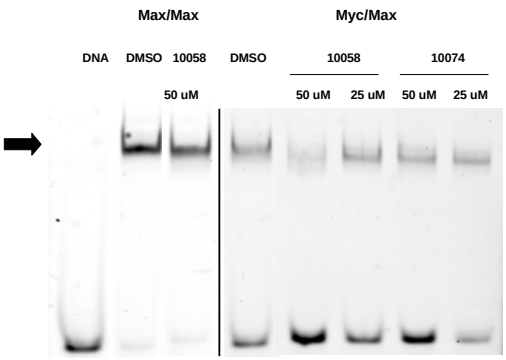

Fig. 2. EMSA assay for inhibition of DNA binding by c–Myc-Max compounds. Purified, recombinant His6-tagged proteins (each >90% pure) (ref. 5) consisted of the bHLH-ZIP domain of human c-Myc (93 amino acids) and full-length human Max, either the 160 amino acid version [Max(160)], which binds DNA as a homodimer, or the 151 amino acid version [Max(151)], which does not bind DNA as a homodimer, but does bind it as a heterodimer in association with c-Myc (5). 25 ng of each purified protein was incubated with approximately 50 pg of the above-described ^{32}P-labeled oligonucleotide (sp. act. approx. 5 x 10^8 dpm/μg) as previously

described (5) in the presence of the indicated compound or the equivalent concentration of DMSO vehicle only. The mixture was then subjected to non-denaturing polyacrylamide gel electrophoresis. The bold arrow indicates the presence of the more slowly migrating protein: DNA complexes. Note in lanes 2 and 3, that 50 uM 10058 reduces DNA binding ability of Max(160) by <20%In contrast, adding progressively increasing amounts of either 10058 or 10074-G5 reduced DNA binding by c-Myc-Max(151) by up to 80% and 50%, respectively (quantification not shown).

Task 4: to employ computerized "data mining" techniques to determine whether other, as yet untested, but structurally related compounds might be better suited as potential therapeutic agents.

Our results, as well as those of others (6) show the feasibility of disrupting the c-Myc-Max association for therapeutic benefit. However, the relatively high concentrations needed to achieve these results (e.g. low μM range) make it unlikely that any of these, in their current state, represent viable therapeutic agents. We believe nonetheless that, as first generation compounds, they can be used as starting points for the rational design of new, more potent compounds.

To illustrate this point, we used *in silico* screening to query a new, small, library of 5040 compounds (ChemDiversity, Inc.) using the ChemFinder program (CambridgeSoft Life Sciences Enterprise Solutions). We initially asked if we could identify structural analogs for compound 10058 containing modifications of only the 6-member ring. 10 such analogs were identified and tested in the yeast 2-hybrid assay. As seen in Fig. 3, these compounds showed a range of activities when compared with the parental compound although none was as effective.

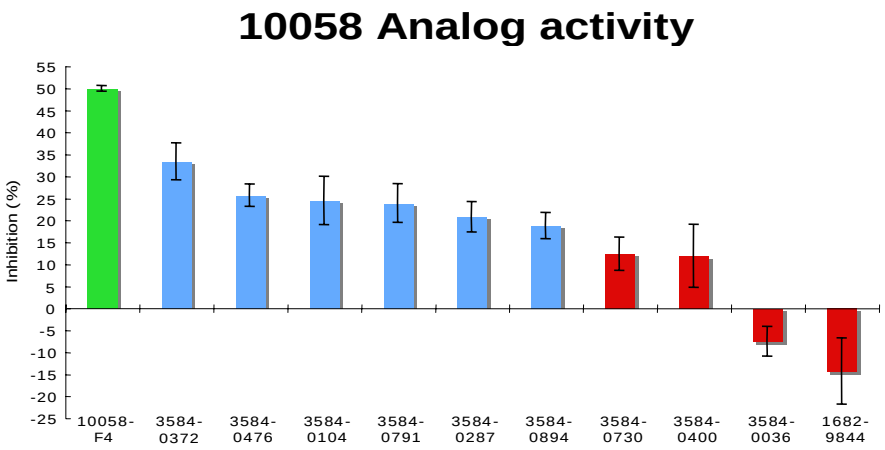

Fig. 3. Activities of analogs of compound 10058 identified using the ChemFinder program. Each of these was then compared to the parental compound for its ability to inhibit the c-Myc-Max interaction using the yeast two-hybrid assay. In these experiments, the conditions of the assay were adjusted so as to reduce its sensitivity, thus potentially allowing for the identification of compounds with activities greater than that of 10058. Thus, 10058 itself showed only a 50% inhibition of β~galactosidase (green bar). The results shown are the average of triplicate experiments +/- 1 standard error. The experiment was also repeated on at least two other occasions with similar results (not shown). Note that in this limited screen, four analogs were identified whose activities were at least 50% that of 10058 whereas two had intermediate activity (blue bars). Four were inactive ≤ 10% activity-red bars).

Although this screen was necessarily limited in scope by virtue of the small size of the screened library, a sufficient number of analogs were found so as to allow some preliminary structure-activity relationships (SARs) to be derived. It can be seen in Fig. 4, for example, that activity is generally retained when the benzene ring of the parental compound is substituted with ether-linked phenyl groups. In addition, whether these groups are in the ortho (0372), meta (0287), or para (0104 and 0791) position of the ring, or whether they consist of mono- (0104), di- (0287 and 0372), or tri- (0791) ethers appears not to affect activity. This suggests that relatively bulky side-

chain additions can be made to the benzene ring without significantly altering activity. The finding that activity is also retained when a meta-NO2 group is added (0372) further underscores the structural plasticity of the benzene ring. On the other hand, the structure is not completely immune to alteration and certain substitutions result in a loss of activity. For example, the addition of a combination of halogen and hydroxyl groups to the benzene ring (0036), or an alkene-linked phenyl group (0730), leads to complete inactivation. These observations suggest that the nature of the benzene ring substitution, rather than its size or position, is the major determinant of activity. Finally, an inactive compound with a rigidified link between the benzene ring and the rhodanine ring (9844), suggests that rotation of the two rings relative to one another is essential for activity.

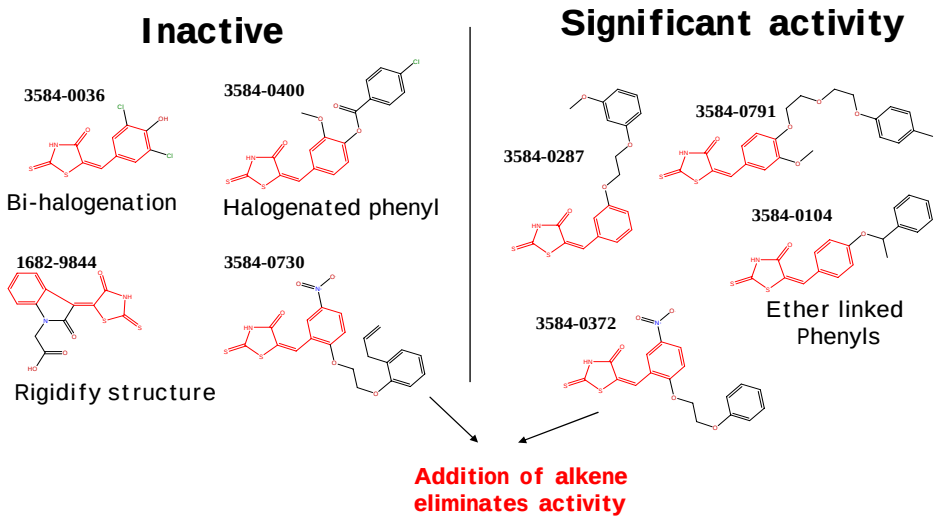

Fig. 4. SARs of select 10058 analogs. The structures of the four most active compounds and the four completely inactive compounds (Fig. 3) are depicted here. Note that the benzene ring can be modified freely with mono-, di-, and tri-ether-linked phenyl groups but that halogen and/or hydroxyl substitutions rendered the compound inactive. Rigidification of the benzene group also leads to a loss of activity.

Based on our *in silico* screening results for 10058 analogs (Figs.3&4), we synthesized 33 additional compounds with alterations in the six member ring only. Several of these (Fig. 5) are particularly critical for addressing SAR predictions arising from the evaluation of the compounds shown in Fig. 4. For example, compounds 12-Rh, 14-Rh, and 23-25-Rh allow us to determine how halogenation of the 6 member ring affects activity, whereas compounds 3-Rh, 26-Rh, 27-Rh, 29-Rh, and 31-Rh can be used to examine the effect of hydroxylation. Similarly, compounds 17-19-Rh allow us to examine the requirement for the benzene ring itself and thus offer an independent evaluation of the activity of the rhodanine ring. Compound 20-Rh is of interest as it permits verification that rigidification of the bond between the two rings is detrimental as predicted from the evaluation of compound 9844 (Fig. 4). Finally, compound 22-Rh tests the need for a saturated 6-member (cyclohexane) ring as well as an assessment of the former structure's "boat-like" conformation versus the planar structure of the unsaturated benzene ring.

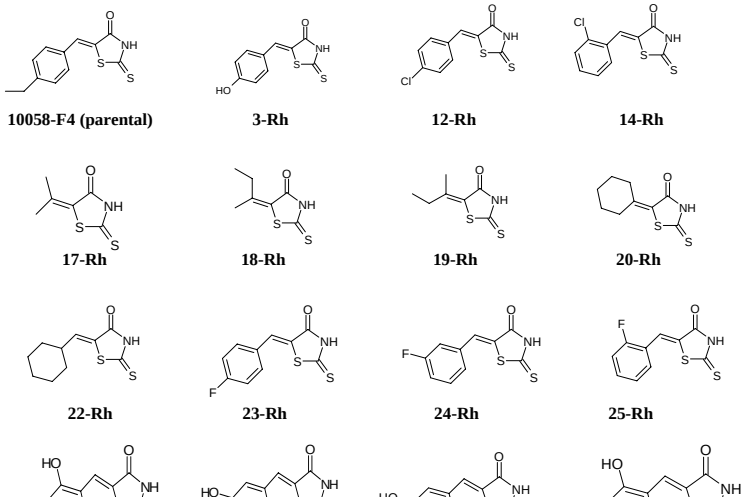

Fig. 5. Structures of a subset of 10058 analogs that have been synthesized. Compounds Rh-12-14 and Rh-18-20 are halogenated at varying positions of the benzene ring, compound Rh-20 has a rigid bond between the 5- and 6-member ring, and compound Rh-22 contains a saturated 6-member cyclohexane ring. Like the parental compound, several of the >30 analogs that have been synthesized are also fluorescent. This will permit a direct assessment of their ability to bind recombinant c-Myc using fluorescence

depolarization. See Appendix 3. for structures of all compounds.

In preliminary experiments, we have examined all of the above compounds in Myc-Max yeast. As seen in Fig. 6, a broad range of activities was observed. For example, chlorination or fluorination, actually improved the activity of the parental compound with the extent of improvement increasing as the modification moved from the ortho- to the para- position. In contrast, hydroxylation at any position severely impaired activity. In combination with the SAR data provided by compound 0036 (Figs. 3&4), this indicates that hydroxylation is the detrimental modification and that it overrides any potential advantage conferred by concurrent halogenation. Another prediction borne out by these more refined studies was that rigidification of the 5-6 member ring linkage (compound 20-Rh) would adversely affect activity. Finally, saturation of the six-member ring also significantly improved

lly consistent with those ciated SAR information.

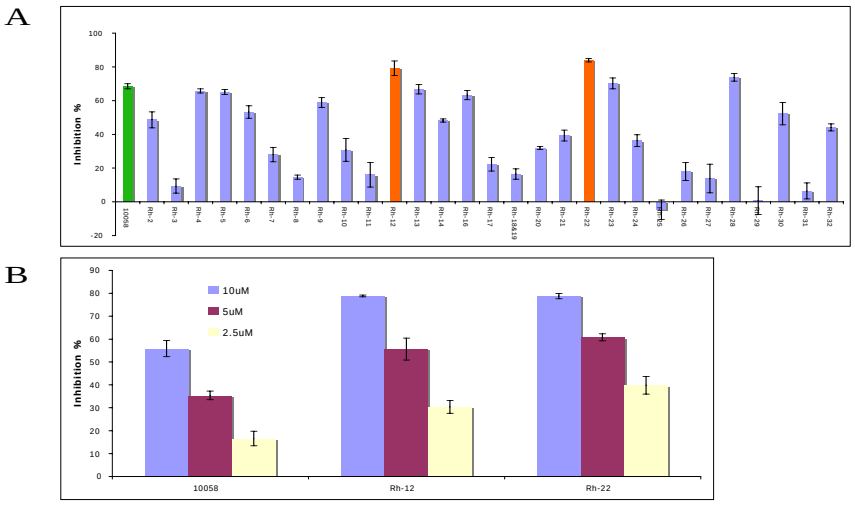

Fig. 6. Activities of rationally designed 10058 analogs in yeast. (A) The compounds depicted in Fig. 13 were tested in the c-Myc-Max yeast two hybrid assay. The parental compound 10058 (green bar) was also tested in parallel. Note that compounds 12-Rh and 22-Rh (red bars) were particularly effective in this assay. Note that 18Rh and 19Rh is a racemate of two stereoisomers of the same compound. (B). Dose-responses of 10058, 12Rh, and 22Rh in the Y2H assay. Note that both of these analogs were 2-4-fold more potent than the 10058 parental compound.

The above results suggest that at least two of the analogs (12Rh and 22Rh) are more potent than the 10058 index compound and that further testing in mammalian cells was warranted. We therefore examined these in the human HL60 pro-myelocytic leukemia cell line, which expresses high levels of endogenous c-Myc due to gene amplification (7). 12Rh proved to be modestly better (Fig. 7). Similar results were seen when the compounds were tested in Rat1a-c-Myc cells, a rat fibroblast line that is transformed by virtue of high level expression of a c-Myc expression vector (2) (not shown).

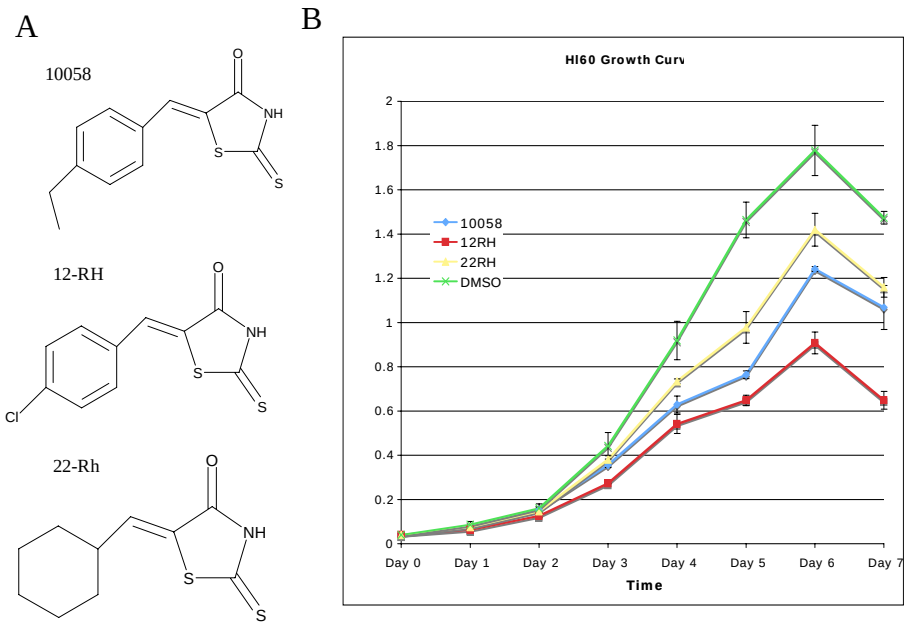

Fig. 7. Efficacy of 12Rh and 22Rh in growth inhibition of HL60 cells. (A). Structures of the parental 10058 compound and its analogs 12Rh and 22Rh. (B). Effects of the three compounds on HL60 cell growth. HL60 cells were plated in triplicate in 6 well plates at an initial concentration of 2 x 10^4 cells/ml. The following day (day 0), the indicated compounds were added at a final concentration of 50 mM. Control cells were incubated in DMSO only (0.1%), which did not appreciably affect the proliferation of viability of the cells. Cell counts were then performed daily. At all times during the course of the experiments cell viabilities remained at >90% as determined by trypan blue exclusion. Note that the concentration of 10058 was lower than that used in Fig. 5, thus accounting for its incomplete effect on proliferation.

Key Research Accomplishments

In summary, we have identified a series of low molecular weight, cell-permeable compounds, which specifically prevent and/or disrupt the association between c-Myc and Max. These compounds can inhibit the interaction between c-Myc and Max in yeast and the growth of cells that express high levels of c-Myc. We have also identified or synthesized a number of "2nd generation" analogs based on modifications of the 6 member ring of one parental compound (10058). Based upon the activity (or lack thereof) of these compounds, we are formulating rules that explain the observed SARs. We expect that such information will be of significant predictive value in the development of even more potent and specific inhibitors.

Plans for the Next Grant Period

1. Further optimization of 10058. Our studies to date indicate that parental compound 10058 binds directly to c-Myc and disrupts its interaction with Max. Computer-assisted and rational structure design approaches have identified several "2nd generation" compounds, with structures based on that of 10058, that have shown increased potency. So far, these structural changes have involved only the 6-member ring of 10058. Over the course of the next year, we plan to test a series of 5-member (rhodanine) ring modifications while holding the original 6 member ring structure constant. Preliminary *in silico* screening as descibed above has identified over 100 such compounds and our collaborator Dr. Metallo has synthesized a number of additional ones. Our preliminary results with a small number of these in yeast, HL60, and Rat1a-c-Myc cells indicates that several are up to 4-times as potent as 10058. Thus we plan a more comprehensive screen to complete the evaluation of all compounds currently at our disposal.

2. Design of "3rd generation" 10058 analogs. Having identified the modifications of both the 5- and 6-member rings of compound 10058 that lead to improved potency in the above-described assays, we

will synthesize so-called "3rd generation" compounds in which optimized 5- and 6-member ring structures will be chemically combined in one molecule. We hypothesize that such structures will demonstrate even greater potency than seen in compounds with only single ring modifications. We believe we are quite close to being able to synthesize these structures and hope to begin screening them within the next 4-6 months.

3. Identification of the binding site for compound 10058. As shown in Fig. 1, we have been able to develop a sensitive, simple, and rapid assay to quantify the binding of compound 10058 to the bHLH-ZIP domain of c-Myc. This suggests that it might be possible to utilize this assay as a means of identifying the specific amino acid residue(s) within this region to which the compound binds. Our rationale for doing this is based upon the idea that if we can identify the binding sites for two such compounds (for example 10058 and 10074-G5), it should be possible to link together their optimized 3rd generation derivatives. The length of the linker would be determined by the distance between the relevant amino acid residues based upon the known c-Myc-Max co-crystal structure (8).

There are several reasons why joining together two c-Myc-Max compounds might prove superior. First, given the proper spacing between the two moieties, the binding of one to its cognate site on the protein's dimerization interface should increase the local concentration of the second compound at or near its own cognate site, thus increasing the probability of binding and allowing the compounds to be used at lower concentrations than would otherwise be effective. Second, the linking of two molecules might reduce or eliminate their potential for non-productive interactions with one another via hydrogen bonding, van der Waals, or hydrophobic interactions. Third, linked compounds might be expected to have fewer non-specific or "off target" effects. Finally, several examples of such covalent linking leading to cooperative effects have already been reported (9, 10). The strategy of multivalent binding (multiple ligands binding simultaneously to a single entity is ubiquitous in nature and has been exploited in multiple synthetic systems (for review see [11]). Of particular interest is an example in which two ligands with micromolar affinity were linked to form a nanomolar affinity ligand of FKBP (12).

In order to identify the amino acid residues necessary for binding by 10058, we have performed random mutagenesis of the 85 amino acid long human c-Myc bHLH-ZIP dimerization/DNA binding domain using an error-prone DNA polymerase. Conditions were chosen so as to introduce an average of one mutation per molecule of the final product. The mutagenized fragments were then cloned into a bacterial expression vector (QE9, Qiagen, Inc. Valencia, CA), in which the bHLH-ZIP domain can be expressed with a His6-tag. A library of several hundred colonies was then picked, grown in 96 well plates, and stored as glycerol stocks. Preliminary sequencing of 20 plasmids has identified a total of 21 different mutations, thus indicating that the library contains a large number of random mutations to perform the proposed studies. Over the next year, we will purify these proteins and evaluate them in the fluorescence depolarization assay. Those failing to bind 10058 will be further examined for their ability to dimerize with Max and to bind DNA. We anticipate that these functions will not be affected by the presence of compound 10058 due to its inability to bind to c-Myc. This same approach should be applicable to any compound whose interaction with c-Myc can be measured.

Reportable Outcomes

Publications:

1. Wang, H., Reese, B., Lazo, J.S., Metallo, S.J. and Prochownik, E.V. Rational Design of Improved Low Molecular Weight Inhibitors of the c-Myc Oncoprotein. (manuscript in preparation)

Suibmitted Grants:

Title: "Optimizing low molecular weight inhibitors of c-Myc for cancer chemotherapy"
PI: Edward V. Prochownik, M.D., Ph.D. (PI) and Steven J. Metallo (co-PI)

Funding agency: NIH
Total amount requested: XXXXXXX (direct plus indirect costs)
Period of grant: 7/1/06-6/30/11

Conclusions

Over the past year, we have begun to refine the relationships between the structure and function of low molecular weight compounds which inhibit the interaction between c-Myc and Max. Starting with one originally identified parental compound (10058) of low potency, we have developed an as yet incomplete set of rules to predict how particular modifications of its 6-member ring will affect activity. Preliminary data suggest that compound efficacy can be significantly enhanced through modifications of the 5-member ring as well. Over the next year, we plan to define the optimized structures of these two modifications and determine how, in combination, they affect activity. We hypothesize that optimizing both rings will ultimately lead to a significant enhancement in potency over that seen with either the original parental compound or any of the single ring analogs. In other work, we have shown that a simple assay fluorescence depolarization assay can be used as a sensitive means of quantifying the interaction between certain of our compounds and c-Myc. This not only establishes that these compounds bind to c-Myc directly but promises the availability of a simple assay to identify the sites on c-myc to which compounds bind. Such information could be used to link together two compounds that bind to different sites within the c-myc dimerization domain to produce a new compound capable of synergistic binding.

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